

Production of Prednisolone by *Pseudomonas oleovorans* Cells Incorporated Into PVP/PEO Radiation Crosslinked Hydrogels

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In order to rise the yield of prednisolone from hydrocortisone, the *Pseudomonas oleovorans* cells were entrapped into radiation crosslinked poly (vinyl pyrrolidone)/poly(ethylene oxide) (PVP/PEO) hydrogel of different gel contents. The factors affecting the gel content and swelling behavior of the polymeric gel, such as polymer composition, polymer blend concentration, and irradiation doses, were investigated. The formation of gels having a good strength with the ability to retain a desirable amount of water in their three-dimensional network can be achieved by using PVP/PEO copolymer of composition (90 : 10) and concentration of 15% prepared at 20 kGy irradiation dose. At these conditions the prepared hydrogel is considered the most favorable one that gave the highest hydrocortisone bioconversion and prednisolone yield, 81% and 62.8%, respectively. The improvement of prednisolone yield was also achieved by increasing substrate concentration. Maximum hydrocortisone bioconversion (86.44) was obtained at 18 hours by using substrate concentration of 30 mg. Reusability of immobilized *Pseudomonas oleovorans* entrapped into PVP/PEO copolymer hydrogel was studied. The results indicated that the transformation capacity of hydrocortisone to prednisolone highly increased by the repeated use of copolymer for 4 times. This was accompanied by an increase in prednisolone yield to 89% and the bioconversion of hydrocortisone was 98.8%.

INTRODUCTION

The method of microbiological transformation of steroids specially based on enzymatic 1,2-dehydrogenation of pregnane derivatives is considered of practical importance because dehydrogenated derivatives of corticosteroids are usually more effective than their precursors in treating rheumatism, unspecific infectious polyarthrititis, and bronchial asthma disease [1, 2, 3]. The use of microorganisms for this purpose is preferred to chemical transformation processes when high specificity is required to attack a specific site on the substrate and to prepare a single isomer of a product. The introduction of this microbial transformation reduced the steps in the synthesis of prednisolone and dropped the production cost. However, by using free microbial cells, reaction times and minimization of the cell lost in the product are not easily controlled. Therefore, immobilization of microbial cells especially used for 1,2-dehydrogenated steroids substrates has attracted considerable interest in recent years. In general, entrapment of microbial cells via natural and synthetic polymeric hydrogels has been employed for immobilization of cells [4, 5].

The main advantages of an immobilized cell compared to a free one are: easy separation from reaction mixture, providing the ability to control reaction times, reuse

of cells for many reaction cycles, lowering the total production cost of cell-mediated reactions, and ability of cells to replace multiple standard chemical steps and provide pure products [2, 6, 7, 8, 9].

Microbial cells can be immobilized on various polymeric entrapment matrices. The choice of the three-dimensional network matrix is very important for the good performance of an immobilized cell system. It is then desirable that a cell carrier possesses large surface area, permeability, hydrophilic character, chemical, mechanical, and thermal stability, suitable shape and particle size resistant to microbial attack, regenerability, and also insolubility. One of the most promising materials for such purpose is the hydrogels. There are different commonly used entrapment hydrogel matrices such as polyacrylamide and hydroxyethyl methacrylate for cell immobilizing. Noticeable interest in the application of radiation techniques to obtain hydrogels for different purposes began in the late sixties [10]. A radiation technique seems to be promising for preparation of hydrogels, because a polymer swollen state undergoes crosslinking on irradiation to yield a gel-like material containing chemically stable C-C bond. This polymer is not contaminated with foreign additives, loses its ability to dissolve in its customary solvents, and its mechanical properties sharply grow [11].

Poly(vinyl pyrrolidone) (PVP) is an example of polymer applied for the synthesis of a hydrogel to be used in different biomedical applications [12, 13, 14]. PVP hydrogels can be obtained by γ -irradiation of PVP water solutions. The physical and mechanical characterizations of the resultant polymer depend on radiation dose as well as the presence of additives in the solution. Poly(ethylene oxide) (PEO) is an example of additive hydrogel which has a good strength and has the ability to increase the elasticity of PVP and retain much water in its three-dimensional network structure [15, 16]. Moreover, the PVA and PEO polymers to be used as a matrix for cell immobilization have the advantage that they are nonionic. The consequence is that the properties of the cells are only minimally modified in the presence of this gel matrix. At the same time, neither the diffusion of the charged substrate nor that of the products is affected.

In this respect, the aim of the present work was to study the possibilities for applying PVP/PEO copolymer hydrogel as entrapment for whole cells immobilization of *Pseudomonas oleovorans* strain with the purpose of increasing the efficiency of hydrocortisone transformation and reaching high operational stability of the biocatalysts obtained in a repeated batch process for production of prednisolone. The effect of polymer composition, polymer blend concentration, total absorbed γ -irradiation dose, and hydrocortisone concentration at different degrees of bioconversion time on prednisolone production was studied. The reusability of poly PVP/PEO immobilized cells was also investigated.

MATERIALS AND METHODS

Chemicals

Cortisol [(hydrocortisone) (11 β , 17 α , 21-trihydroxy preg-4-ene-pregnen-3, 20-dione) Kendall's compound], prednisolone (11 β , 17 α , 21-trihydroxy-1, 4-pregnadiene-3, 20-dione) were supplied by Sigma Chemical Co, USA/Canada; Poly(vinyl pyrrolidone) PVP (MWt 13 000 000, K₈₅₋₉₅) and poly(ethylene oxide) PEO (MWt 600 000) were supplied by Arcos Organics Co, Belgium.

Microorganism

Pseudomonas oleovorans was provided by ATCC 29347. *Pseudomonas oleovorans* was grown on the broth medium containing (g/L): glucose, 10.0; peptone, 6.0; yeast extract, 3.0; meat extract, 1.5. The pH of the medium was adjusted to 6.5 with NaOH or HCl [17]. The previous medium was sterilized at 121°C for 20 minutes. The incubation period continued for 24 hours and then the medium was transferred on slop agar medium of the same composition. The organism was maintained on this medium, stored at 4°C, and subcultured monthly.

Medium and culture conditions

Two milliliters of cell suspension of 7-day old culture of *Pseudomonas oleovorans* were allowed to grow

in 250 mL Erlenmeyer flask containing 50 mL of sterile medium, as described above, at 30°C for 48 hours with shaking at 200 rpm. The inoculated medium was centrifuged using cooled centrifuge (4000 rpm) at -4°C for 15 minutes. The harvested cells were washed with 0.01 M sodium phosphate buffer pH (7.0) and became ready to be incorporated into PVP/PEO copolymer hydrogels.

Cell immobilization

Preparation of hydrogels

Unless otherwise stated, PVP/PEO aqueous solutions (90 : 10) (PVP/PEO) copolymer composition of 15% concentration was prepared using an autoclave at 121°C for 120 minutes. The aqueous solutions were poured in glass tubes. The nitrogen gas was passed through the solutions for 24 hours to remove the dissolved oxygen. Then, 2 milliliters of cell suspensions were mixed with prepared PVP/PEO blends and homogenized with shaking technique. Then the PVP/PEO blends were exposed to gamma irradiation using a Co⁶⁰ gamma cell (20 kGy) (unless otherwise stated) at a fixed dose rate of 8 kGy/h. The resultant hydrogels were cut into discs, approximately 1–3 mm in diameters and 2–3 mm in thickness. The discs were washed several times with 0.01 M sodium phosphate buffer (pH 7.0). All the above-mentioned procedures were performed under sterile conditions [21].

Irradiation process

Mixtures were irradiated to 20 kGy (unless otherwise states) at 35°C in a Co⁶⁰ Russian-type γ -irradiator at a fixed dose rate of 8 kGy/h.

Evaluation of immobilized cells bioconversion

Unless otherwise mentioned, twenty grams (wet weight) of PVP/PEO hydrogels discs of a ratio (90 : 10) (w/w) at 15% polymer blend concentration prepared by γ -radiation polymerization at 20 kGy, immobilized by two milliliters of *Pseudomonas oleovorans* cell suspension were cultured in a 250 mL Erlenmeyer flask containing 50 mL of sterile medium containing (g/L): glucose, 10; peptone, 5, and beef extract, 3 [17]. The pH of the medium was adjusted initially by HCl or NaOH to 7.0, and then 30 mg of hydrocortisone dissolved in 96% ethanol were added. The bioconversion was carried out at 30°C on a rotatory shaker at 200 rpm for 18 hours.

Production of prednisolone using 2L fermentor

2L fermentor was from New Brunswick Co, Inc (Edison, NJ). 1300 mL of modified production medium containing (g/L): glucose, 10; peptone, 5, and beef extract, 3; pH 7 were inoculated by immobilized *Pseudomonas oleovorans* of PVP/PEO hydrogel discs [20 gm(wet weight)/50 mL medium]. Then 30 mg of hydrocortisone dissolved in 96% ethanol were added. The bioconversion was carried out at 30°C for 18 hours with optimal rate of agitation 200 rpm, optimal rate of aeration was 1 volume of sterile air per volume of production medium per

minute (V/V/min). Exhaust gas escaping was applied under gauge pressure of 11.5 cm water column. The foaming was controlled by the addition of 1 mL of sterilized paraffin oil. Thereafter, the transformation medium was withdrawn, PVP/PEO hydrogel discs were washed with nutrient medium and used for further transformation procedure. The recyclization was carried out for 9 cycles by using the same procedure and the same medium which was previously described.

Extraction and analytical procedure

Samples were extracted by shaking with 100 mL of chloroform (Merck, Germany). The extraction was repeated three times. The combined chloroform extracts were washed with one half of their volume distilled water, dried over anhydrous sodium sulfate, and filtered. The filtrate was distilled to give a semisolid residue (test materials). The test materials were assayed for hydrocortisone and prednisolone contents by HPLC (thermo separation product, Aldrich), with the following conditions: column (spherisorb, 250 mm length and 4.6 mm diameter), mobile phase (distilled water 65% + tetrahydrofurane 35%), flow rate 1 mL/s, and a UV detector at 254 nm. All steroids reference standards were obtained from Sigma Co, USA, and all reagents were HPLC grade.

Gel determination

In order to extract the insoluble parts of the hydrogels, that is, the gelled part, the prepared hydrogels were soaked in water for 48 hours at 100° C, then they were taken out and washed with hot water to remove the soluble part, dried and weighed. The gel percent in the hydrogel was determined from the following equation:

$$\text{Gel}(\%) = \frac{WE}{Wg} \times 100, \quad (1)$$

where *WE* and *Wg* are dry hydrogel weights after and before extraction, respectively [18].

Swelling measurement

The equilibrium swelling time of hydrogels depends on their gel content and crosslinking density which are correlated to their preparation conditions. Therefore, the water content of the hydrogel formed by irradiation was determined by immersing the hydrogel in production medium at 30° C for 24 hours (24 hours are a sufficient and suitable time for equilibrium swelling of hydrogels of different crosslinking degrees) and then weighed. The water content was calculated based on the weight difference of the dry and swollen samples by using the following equation:

$$S_w(\%) = \frac{W_s - W_0}{W_0} \times 100, \quad (2)$$

where *W₀* and *W_s* are the weights of gel in the dry and swollen states, respectively [16].

TABLE 1. Gel content and swelling behavior of PVP/PEO prepared at different copolymer compositions (Wt/Wt), irradiation dose 20 kGy, PVP/PEO copolymer concentration 10%.

PEO/PVP composition (Wt/Wt)	Gel content (%)	Water uptake (%)
0 : 100	95	2450
10 : 90	92	2640
15 : 85	87	2800
60 : 40	82	2980
40 : 60	80	3090
100 : 0	78	3200

The total uncertainty for all experiments ranged from 3%–5%.

RESULTS AND DISCUSSION

Effect of different compositions of PVP/PEO on the hydrocortisone bioconversion by immobilized *Pseudomonas oleovorans*

PVP/PEO copolymer hydrogels were used for immobilization of the cells responsible for biotransformation of hydrocortisone to prednisolone. Therefore, the effect of different PVP/PEO compositions on their gel content and swelling property was studied and it is shown in Table 1. It is clear that the copolymer swelling property increases with the increase of the PEO ratio in PVP/PEO polymer blend feed solution. However, the copolymer gel content increases as the PEO ratio in PVP/PEO polymer blend feed solution decreases. The results assumed that the PEO possesses highly flexible and mobile chains. The excess of PEO in the blend feed solution reduces the formation of crosslinking network of the copolymer.

The effect of PVP/PEO of different compositions on the prednisolone production yield was investigated and it is shown in Figure 1. The prednisolone yield percentage reached the maximum value (51%) when cells were entrapped in PVP/PEO of composition (90 : 10) and this is accompanied with an increase in its productivity (2.16 mg/L/h). Increasing PEO content in PVP/PEO to greater than 10 leads to decrease in prednisolone yield (40, 33, 29, and 16%) for PVP/PEO of compositions (85 : 15), (60 : 40), (40 : 60), and (0 : 100), respectively, and this also was accompanied with a decrease in prednisolone productivity, 1.67, 1.39, 1.25, 0.68 mg/L/h, respectively.

The lower concentration of PEO gel probably causes a suitable decrease in the crosslinking density of PVP network. This is due to the flexibility and high mobility of PEO chains which retard the formation of high-degree crosslinked PVP/PEO copolymer, resulting in the formation of gels having a good strength with the ability to retain a desirable amount of water in their three-dimensional network structure and therefore becoming more porous gels so that, the Δ'-dehydrogenation process

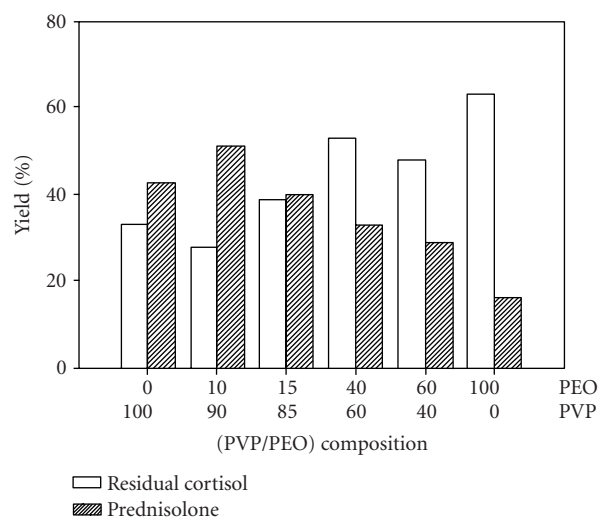


FIGURE 1. Effect of PVP/PEO of different compositions on the prednisolone production yield. PVP/PEO irradiation dose 20 kGy; copolymer concentration 10%.

TABLE 2. Gel content and swelling behavior of PVP/PEO prepared at (90 : 10) copolymer composition (Wt/Wt) and dose of 20 kGy.

Polymer blend concentration (%)	Water content (%)	Gel content %
5	2900	79
10	2640	92
15	1820	95
20	1220	95

would be activated smoothly. On the other hand, the increase in the concentration of PEO gel more than one tenth of PVP may give a hydrogel with high elasticity, low gel content, and high swelling property (Table 1) due to the decrease in the gel content and crosslinking density of PVP hydrogel. These seem to be undesirable for the fermentor and bioreactors applications [14, 15].

Effect of different PVP/PEO copolymer concentrations on their gel content, swelling properties, and hydrocortisone bioconversion

Effect of different concentrations (5, 10, 15, and 20) of polymer blend on the gel content and swelling property of PVP/PEO copolymer was studied and it is shown in Table 2. As the polymer blend concentration increases, the copolymer gel content increases and its ability to swell decreases. By increasing the polymer blend concentration, its viscosity increases. The high viscosity of solutions increases the radical ability to survive a long period during the irradiation process, resulting in high-degree crosslinked PVP/PEO.

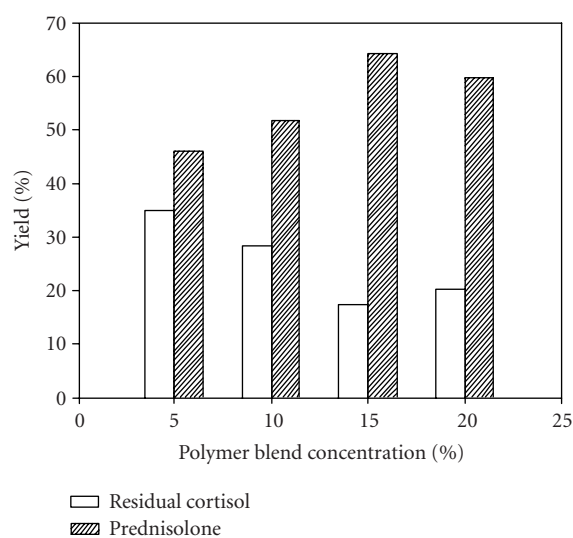


FIGURE 2. Effect of different concentrations (5%, 10%, 15%, and 20%) of PVP/PEO copolymer, obtained by γ -irradiation at dose of 20 kGy, on the Δ' -dehydrogenation of hydrocortisone bioconversion and the yield percentage of prednisolone, copolymer composition (90 : 10)(Wt/Wt).

Effect of different concentrations (5, 10, 15, and 20) of PVP/PEO copolymer, obtained by γ -irradiation at dose of 20 kGy, on the Δ' -dehydrogenation of hydrocortisone bioconversion and the yield percentage of prednisolone was investigated and it is shown in Figure 2. It is observed that as the copolymer concentration increases, the prednisolone yield and its productivity increase reaching the maximum (62.8% and 2.61 mg/L/h) at 15% copolymer concentration. However, the concentrations of PVP/PEO lower than the optimum (5, 10%) and higher than the optimum (20%) give lowest prednisolone yield and productivity (46, 51%), (59%), and (1.92, 2.16, and 2.49 mg/L/h), respectively. The swelling ratio of PVP/PEO prepared at low concentration (5, 10%) is higher than that prepared at high concentration [19].

At higher swelling ratio (5, 10%), the cells released from the gel to the medium easily and are so affected by all the factors affecting the intact cells (acidic, alkaline, saline media and toxicity of ethanol dissolved in its substrate), resulting in a decrease in the Δ' -dehydrogenase process activity. However, at lower swelling ratios (20%), the crosslinking density and the mechanical strength of hydrogel increase, leading to a decrease in the permeability of the substrate and the product. As a result, the Δ' -dehydrogenase process activity decreases. Therefore, the copolymer concentration of (15%) is considered the most favorable blend concentration, which gave the highest hydrocortisone bioconversion, prednisolone yield, and productivity (81.9%, 62.8%, and 2.61%), respectively, [19, 20].

Figures 3a, 3b, and 3c represent the surface topography of PVP/PEO of (90 : 10) copolymer composition, prepared at different blend concentrations 10, 15, and

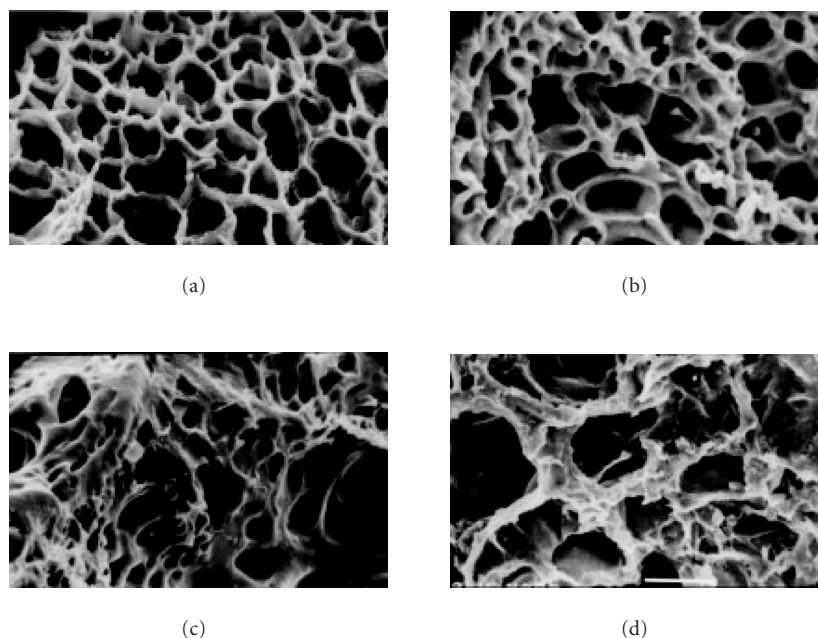


FIGURE 3. Figures 3a, 3b, and 3c represent the surface topography of PVP/PEO of (90 : 10) copolymer composition, prepared at different copolymer concentrations (Figure (a) 10%, (b) 15%, and (c) 20%). Figure (d) represents the surface topography of PVP/PEO of (90 : 10) copolymer composition, prepared at a copolymer concentration of 15% after soaking it in the reaction medium for a long time (48 hours), irradiation dose of copolymers was 20 kGy.

TABLE 3. Gel content and swelling behavior of PVP/PEO of (90:10) composition (Wt/Wt) at different irradiation doses; copolymer concentration (20%).

Irradiation dose (kGy)	Water content (%)	Gel content (%)
10	1840	85
20	1220	95
30	980	98

20% and irradiation dose of 20 kGy. It is clear that as the blend concentration increases, the pore size decreases due to an increase in copolymer crosslinking density.

Effect of the degree of crosslinking of PVP/PEO hydrogels prepared at different irradiation doses on the hydrocortisone bioconversion

The effect of irradiation dose on the degree of crosslinking and swelling property of PVP/PEO hydrogels was investigated and is shown in Table 3. By increasing the irradiation dose, the gel content and crosslinking degree increase resulting in a decrease in copolymer swelling property. As the irradiation dose increases, the polymer free radicals increase and consequently the degree of crosslinking and gel content increase.

Bioconversion of hydrocortisone to its derivative prednisolone by immobilized cells is greatly affected by the total γ -irradiation dose adsorbed by PVP/PEO hydrogels. Table 4 shows the relationship between hydro-

cortisone bioconversion and prednisolone yield against PVP/PEO copolymers prepared at different irradiation doses (10, 20, and 30 kGy). The prednisolone yield reached the maximum value (62.8%) when γ -radiation of 20 kGy was used for PVP/PEO copolymerization process. However, by using PVP/PEO copolymers prepared at 10 and 30 kGy, the prednisolone yields were 44.4 and 48.7%, respectively.

At low irradiation dose, the degree of crosslinking of the polymer matrix-entrapped cells is low. Therefore, the cells easily release from the polymer matrix to the medium giving the lowest hydrocortisone bioconversion [4]. At the high irradiation dose (30 kGy) crosslinking degree, network density and gel content of PVP-copolymer hydrogels is high. These reduce the copolymer swelling values and constantly the diffusion rate of substrate and product through the copolymer matrix decreases resulting in reduction in the hydrocortisone bioconversion [21, 22]. Hence, 20 kGy irradiation dose is considered the most favorable dose suitable for crosslinking copolymerization process of PVP-PEO matrix-entrapped cells, which gave the highest hydrocortisone bioconversion (81.9%) and higher prednisolone productivity (2.61 mg/L/h).

Δ' -dehydrogenation of different hydrocortisone concentration at different time of fermentation by immobilized *Pseudomonas oleovorans*

The improvement of prednisolone yield was achieved by increasing substrate concentration to some limit. The

TABLE 4. Effect of the degree of crosslinking of PVP/PEO hydrogels prepared at different irradiation doses on the hydrocortisone bioconversion of PVP/PEO of (90:10) composition (Wt/Wt), copolymer concentration (20%).

Adsorbed γ -rays (kGy)	Transformation mixture		
	Residual cortisol (%)	Prednisolone	
		Yield (%)	Productivity (mg/L/h)
10	49.0	44.4	1.85
20	17.5	62.8	2.61
30	31.0	48.7	2.03

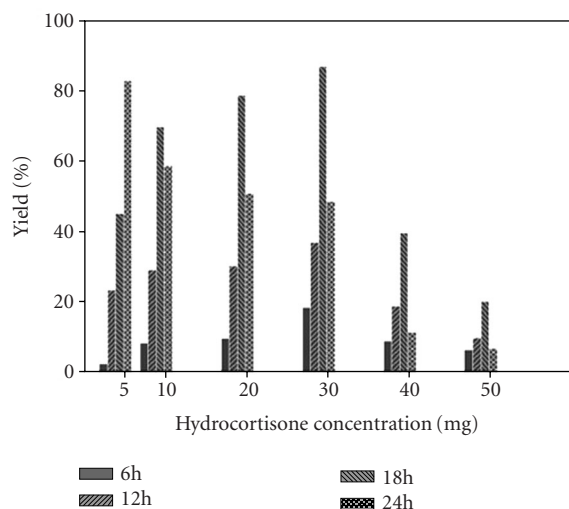


FIGURE 4. Δ' -dehydrogenation of different hydrocortisone concentrations at different times of fermentation by immobilized *Pseudomonas oleovorans* PVP/PEO copolymer prepared at (90:10) PVP/PEO copolymer composition, (15%) blend concentration, and 20 kGy γ -irradiation dose.

degrees of bioconversion of various initial concentrations of hydrocortisone (10, 20, 30, 40, 50 mg/50 mL) after 6, 12, 18, and 24 hours, performed in shaken flasks are shown in the histogram in Figure 4; the maximum hydrocortisone bioconversion (86.44) was obtained at 18 hours by using substrate concentration 30 mg. Increasing the substrate concentration to 40 and 50 mg/50 mL medium led to a progressively lower conversion of hydrocortisone to 39.22 and 19.67%, respectively at 18 hours. The histogram also shows that the conversion percentage measured at 30, 40, and 50 mg/50 mL medium at 24 hours becomes lower (48.13, 10.94, and 6.43%) compared with the conversion percentage measured at 10 and 20 mg at the same time (58.33 and 50.49%), respectively. This is due to that the higher concentration of substrate may block the pores of the hydrogel and also hinder the exchange of oxygen and substrate diffusion through the hydrogel [2, 22, 23, 24]. In general, the conversion percentages measured at 10, 20, 30, 40, and 50 mg/50 mL medium at 18 hours were higher than those obtained at 24 hours. This phenomenon was demonstrated by some investiga-

tors [25, 26, 27, 28]. The equilibrium may be displaced in the direction of oxidation or reduction of steroids according to the conditions of the fermentation, specially time of fermentation and substrate concentration. The same results were obtained in previous work [22].

Stability studies

Reusability of immobilized *Pseudomonas oleovorans* in PVP/PEO hydrogel.

The economics of an immobilized cell technology depends on the lifetime of the biocatalyst. The reusability of immobilized cells entrapped into PVP/PEO copolymer prepared at (90/10) PVP/PEO copolymer composition, (15%) blend concentration, and 20 kGy γ -irradiation dose was studied by using 2L New Brunswick fermentor instead of shaken flasks. The analyses of the sample (30 mg/50 mL medium) were carried out each 18 hours. The results presented in Figure 5 indicated that the transformation capacity of hydrocortisone to prednisolone highly increased by the repeated use of polymer blend of composition (15%) for 4 times. This is accompanied by an increase in prednisolone yield to (89%) and the bioconversion of hydrocortisone is (98%). Meanwhile, the immobilized cells reused more than 4 times slightly decrease the prednisolone yield to (68.8%) and (66.5%) at the 5th and 6th cycles, respectively. But the immobilized cells reused more than 6 times highly reduce the prednisolone yield percentage as shown in the histogram up to the 9th cycle. From the above results, we concluded that the cells probably grow in the gel so that the prednisolone yield percentage gradually increases [7, 24]. The repeated use of immobilized cells after the 4th cycle gradually decreases the prednisolone yield percentage and its productivity to (68.8–31.2%) and (3.8–1.7 mg/L/h), respectively, and this may be due to the lyses of the cells, and thus the density of the immobilized cells becomes lower and so led to lower in the cells multiplication and so decreases in the activity of the Δ' -dehydrogenase [29].

On the other hand, it was found that by soaking the gel in the reaction medium for a long time (48 hours), its pore size increases (Figures 3b and 3d). This observation explains the reduction of prednisolone yield after using the cell-entrapped copolymer system for several times. As the pore size increases, the ability for entrapped cell to scrape outside increases, resulting in a decrease in prednisolone yield.

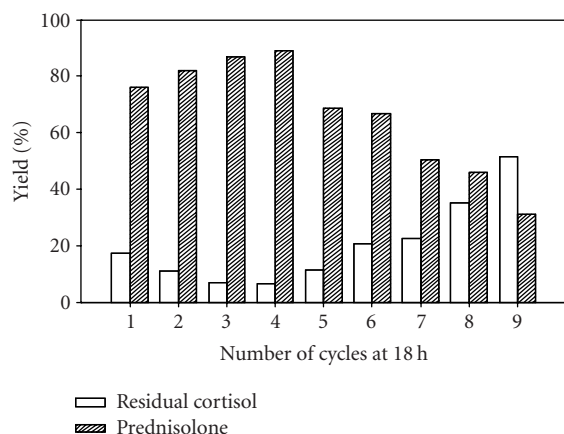


FIGURE 5. Reusability of immobilized *Pseudomonas oleovorans* in PVP/PEO hydrogel copolymer prepared at (90:10) PVP/PEO copolymer composition, (15%) copolymer concentration, and 20 kGy γ -irradiation dose.

CONCLUSION

Radiation crosslinking PEO/PVP copolymer hydrogels were successfully used for immobilization of *Pseudomonas oleovorans* cells responsible for biotransformation of hydrocortisone to prednisolone with a sufficiently high activity. Copolymers of suitable crosslinking degree, obtained by using PEO/PVP copolymer of composition (10 : 90) and irradiation dose of 20 kGy, absorb a reasonable amount of water, prevent the microbial cells scarping, and increase prednisolone yield. The prednisolone yield was also improved by increasing the substrate concentration to some limit. Stability studies reflect the capability of the PEO/PVP copolymer to retain the immobilized cells and form a stable system that can be used for regular transformation procedures of hydrocortisone up to 4 times with an increase in prednisolone yield to (89%), and the bioconversion of hydrocortisone is (98.8%). Meanwhile, the immobilized cells reused more than 4 times slightly decrease the prednisolone yield to (68.8%) and (66.5%) at the 5th and 6th cycles, respectively.

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